

The Effects of Stress on Global Cerebral Ischemia

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by

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Abstract

Cardiovascular disease is the leading cause of death and disability in the United States. There is the potential for permanent damage to the brain even among those individuals who are successfully resuscitated. This damage can be exacerbated with the addition of stress. The purpose of this project was to determine the effects that chronic stress have on mice following cardiac arrest with cardiopulmonary resuscitation (CA/CPR). It was hypothesized that mice exposed to chronic stressors would have sufficient levels of glucocorticoids to exacerbate post-CA/CPR behavioral alterations, neuronal death, neuronal morphology, and microglia activation. The mice in the stress groups were subjected to randomly timed (between 00:00 and 14:00 hr) restraint for two weeks, 2 h/d prior to CA/CPR. Forty-eight hours following the last exposure to the experimental stressor, the mice were exposed to global cerebral ischemia followed by reestablishment of blood flow 8 minutes later. Experimental global cerebral ischemia was induced by injecting potassium chloride via a catheter attached to the right jugular vein. After 8 minutes, resuscitation was initiated by injection of epinephrine in conjunction with chest compressions. The core body temperature was manipulated to limit cell damage strictly to the brain by lowering the core to 27°C and maintaining the head at 37°C.

Behavioral responses to CA/CPR were assessed using the elevated plus maze test and the open field test. These tests were used as indices of anxiety-like behavior following CA/CPR.

Hippocampal tissue was collected 24 hours after resuscitation to assess cytokine production. The CA/CPR groups produced significant increases in gene expression of Interleukin-1 β (IL-1) and tumor necrosis factor- α (TNF- α) when compared to the sham-operated (SHAM) counterparts. However, the stress+CA/CPR group means were not significantly different from the CA/CPR groups. In a separate cohort of mice, behavioral analyses were conducted and tissue was collected 5 days post-CA/CPR surgeries. Hematoxylin and eosin stain (H&E) revealed significant increases in neuronal cell death following CA/CPR relative to SHAM, and was further exacerbated by the addition of chronic stress. The microglia activation marker (Mac-1) stain in the CA/CPR group revealed significant increases in activation of microglia when compared to SHAM, but there was no effect of stress on these measures. The Golgi stain with Scholl analyses displayed apical dendritic retraction in the hippocampus relative to the SHAM following CA/CPR. This was exacerbated in the mice that were exposed to chronic stress prior to CA/CPR. This same pattern was observed in reduction of dendritic spine density. Overall, these data suggest that glucocorticoids are a key player in neuroinflammation and immune dysfunction following CA/CPR.

Introduction

An estimated 79,400,000 American adults, or 1 in 3, have at least one or more types of cardiovascular disease [1]. Cardiovascular disease was responsible for 1 in every 5 deaths in the United States in 2004 and the single largest killer of Americans. About every 26 seconds, an American will suffer a coronary event, and about every minute someone will die from one. Additionally, close to 38% of people who experience a coronary attack in a given year will die from it [1].

Cerebral damage, specifically neuronal death is predominately localized to the hippocampus following cardiac arrest with cardiopulmonary resuscitation (CA/CPR), in both clinical and experimental disease. This region of the brain is the most sensitive to lack of oxygen and therefore the one most likely to reveal damage following short durations of ischemia [4]. Additionally, many studies have shown that the reoxygenation during reperfusion produces many reactive oxygen species (ROS) such as hydroxyl radicals and nitric oxide. These ROS contribute to oxidative damage in ischemic tissue and leads to cell death [2] [3].

Extracellular glutamate concentrations increase several-fold in the CA1 region of the hippocampus during cerebral global ischemia [4]. The cell death observed in this area of the brain may be due in part to glutamate excitotoxicity. During ischemia-induced depolarization, large amounts of the amino acid neurotransmitter glutamate are released and received by protein receptors on dendrites. One class of glutamate receptors is the ionotropic receptors such as N-methyl-D-aspartate (NMDA) receptors, which are ligand-gated ion channels.

When glutamate binds to the NMDA receptor, Ca^{++} flows into the post-synaptic cell which then causes it to depolarize and produce another action potential in a new cell body. During an ischemic insult, extracellular concentrations increase which provides ischemia-induced Ca^{++} overload resulting in neuronal cell death [3]. This continuous process along with other factors such as the inability of the Na^+/K^+ ATPase pumps to sufficiently pump K^+ in and Na^+ out due to lack of ATP, are why the cells cannot repolarize. The process of repolarization is essential for homeostasis so the cell bodies can continue to produce action potentials to carry information throughout the body.

The dendritic projections along with the dendritic spines are sensitive to extracellular glutamate concentration. The large increase in the extracellular concentration of this amino acid changes the calcium homeostasis leading to retraction of both dendritic projections and dendritic spines [4]. It has been observed *in vitro* that when dendrites are exposed to toxic levels of glutamate that dendritic spine retraction occurs within minutes, but there is uncertainty as to whether this is a less severe form of neuronal damage or just a protective response to altered extracellular concentrations of neurotransmitters [4].

During the reperfusion process there is accelerated structural damage as a consequence of excessive production of free radicals by lipid peroxidation. Oxygen radicals are extremely reactive species and will oxidize macromolecules such as proteins, lipids and nucleic acids. As a result they can alter the fluidity and permeability of cells' membranes thereby exposing cellular proteins, ion channels, and receptors consequently compromising cell function [3].

Exposure to stress has been hypothesized to exacerbate the immune dysfunction and neuroinflammation following global cerebral ischemia. Stress is a universal condition that affects our daily lives; however, there is variability in the stressor, its severity, coping strategies, and the individual's perception of that stressor as being potentially harmful. So what exactly characterizes stress? A physiological definition is the activation of the hypothalamic-pituitary-adrenal (HPA) axis and/or the sympathetic nervous system in response to a stimulus, thus allowing an organism to adapt physiologically to a perceived threat [5]. Psychologically, a student taking an examination or a husband or wife caring for a spouse with Alzheimer's disease could be an event perceived as stressful [5]. Stress is known to cause the release of several so-called stress hormones, primarily glucocorticoids through activation of the HPA axis in addition to catecholamines via the sympathetic nervous system [6]. When experiencing a perceived stressor, one characteristic is common: raised plasma levels of glucocorticoids. Thus, stress can be operationally defined as increased concentrations of glucocorticoids, cortisol in humans and corticosterone in mice and rats [7].

The brain influences what is perceived as stressful along with the physiological responses that are produced. These stress responses can be adaptive or maladaptive and target the cardiovascular, metabolic, and immune systems along with other systems of the body [8]. The central nervous system controls the HPA axis, which in turn responds to stress by increasing the level of circulating of glucocorticoids stimulated by the release of corticotropin-releasing

hormone (CRH) from the hypothalamus. Glucocorticoids are lipophilic substances that can easily cross the cell membrane and interact with glucocorticoid receptors (GR) in the cytoplasm. Once bound, GRs undergo a conformational change and translocate to the nucleus where they function as a modulator of gene transcription. The cascade of events begin with corticotropin-releasing hormone, a hypophysiotropic hormone released upon activation of the HPA axis, which then stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. Increased plasma levels of ACTH stimulate cortisol or corticosterone secretion from the adrenal cortex [9]. The system is then controlled via a negative feedback loop onto the hippocampus and hypothalamus, where glucocorticoids inhibit further release of CRH and ACTH from the hypothalamus and anterior pituitary gland, respectively. The increased plasma glucocorticoid concentrations increase blood pressure, mobilization of glucose, as well as immunosuppressive activities [10].

Immune function is affected by the neuroendocrine signal CRH being released from the brain. The secretion of this hormone is caused by both psychological and physical stressors. In normal resting state, basal concentrations of CRH, ACTH, and cortisol are released in both a circadian and pulsatile fashion. This basal level of cortisol functions to prevent arterial hypotension and to sustain normoglycemia [11]. On the other hand, activation of the HPA axis as a result of physical and/or emotional stress raises the plasma cortisol levels above the baseline, and typically results in anti-inflammatory / immunosuppressive activities in the periphery. One example of the many

immunosuppressive activities of glucocorticoids can be observed in the inhibition nuclear factor- κ B (NF- κ B). NF- κ B and GR are both transcription factors that modulate the immune/inflammatory function, with opposing actions; NF- κ B induces the expression of pro-inflammatory genes while GR suppresses the actions of NF- κ B [12]. The immune system is affected by both acute and chronic activation of the HPA axis. What is often misunderstood is that stress hormones can be beneficial and adaptive in the short run; in contrast, chronic activation of the HPA axis and its resultant, overproduced hormones has deleterious consequences on the brain and on immune function [5]. More specifically, chronic stress increases the incidence and/or exacerbation of many numerous neurological disorders which include age-related dementia, multiple sclerosis, depression, and Parkinson's disease [8] [13].

It has been well documented that glucocorticoids have immunosuppressive activities in the periphery, but depending on the brain region, chronic exposure to glucocorticoids can actually exacerbate the pro-inflammatory effects of the immune system [10]. One reason for this observation is because cortisol can cause glucose uptake inhibition into neurons. This in itself is not energetically disruptive, but depletes the amount of ATP that the mitochondria are able to produce. As a result, the neuronal cells have less energy for the very costly task of reuptake of increased extracellular concentrations of glutamate after an injury to the central nervous system. Weakening neurons can render them more likely to die from other insults and subsequently release cellular debris such as ATP, glutamate, and calcium. Additionally, the release of pro-

inflammatory cytokines such as TNF- α and IL-1 are observed, thereby sequestering a stronger immune response and causing the activation of microglia and astrocyte proliferation [10].

Stress and cardiac arrest both can have deleterious affects on an individual's homeostasis and together can alter the endogenous physiology of the central nervous system. Because stress impairs neuronal metabolism, stress prior to cardiac arrest may exacerbate the affects of global cerebral ischemia. If this were the case, then we would hypothesize that 14 days of chronic confinement stress prior to cardiac arrest would cause an increase in neuronal cell death, a decrease in spine density, a decrease in arborization length, and increase in inflammatory markers (such as pro-inflammatory cytokines and activation of microglia/macrophages).

Materials and Methods

Animals

The Institutional Laboratory Animal Care and Use Committee of Ohio State University approved all animal procedures in accordance with the guidelines set forth by the National Institute of Health. Adult male C57BL/6 mice (Charles River, Wilmington, MA, USA) were housed individually in polycarbonate cages (28 × 17 × 12 cm) from the onset of the study in rooms maintained at 20° ± 4 °C and relative humidity of 50 ± 5% on a 14:10-h light/dark cycle, with *ad libitum* to tap water and food (LabDiet 5001; PMI Nutrition; Brentwood, MO, USA).

Cardiac arrest/cardiopulmonary resuscitation procedure

Mice were anesthetized with 3% halothane in air, and they were intubated and maintained on 1.5% halothane. A temperature probe was placed in the temporalis muscle on the left side of the head, which was used as an index of temperature. The cortical temperature and temporalis temperature are highly correlated ($r^2 = 0.94215$), which was validated by a previous study in rats [14], over the range of temperatures experienced during our cardiac arrest/CPR and SHAM procedures (24°C to 39.5°C). Therefore the temporalis muscle temperature was used as an index of brain temperature. To maintain body temperature, another probe was inserted into the rectum and provided a feedback to a heating blanket (Harvard Apparatus, Holliston, MA, U.S.A.). A PE10 catheter was inserted into the right jugular vein for administration of potassium chloride (KCl) and epinephrine, respectively. The right femoral artery was exposed and inserted a cannula (Fine Science, Foster City, CA, U.S.A.) which was connected to a blood pressure transducer (Columbus Instruments, Columbus, OH, U.S.A.) to allow continuous monitoring of arterial blood pressure. The intubation tube was connected to a ventilator (Columbus Instruments, Columbus, OH, U.S.A.), and the mice were ventilated with a tidal volume of 150 μ L and a respiratory rate of 155 breaths per minute. The mice stabilized for 10 minutes. During this time period the blood pressure and temperatures were recorded at 1-minute intervals (Fig. 1 – use in presentation). At the end of the acclimation period, circulating cold water was used to decrease the body

temperature to 27°C through a coil system beneath the animal and placement of an alcohol patch on the ventrum. A double lumen coil was placed around the head and filled with circulating water to independently manipulate the body temperature to achieve a brain temperature of 27°C, 37°C, or 39°C. KCl (50.0 µL, 0.5 M, 4°C) was injected via the jugular catheter to induce cardiac arrest, and the animal was detached from the ventilator. Slow rewarming via heating lamp and thermal blanket began when body temperature reached 27°C after approximately 4 minutes of arrest. At 7 minutes 45 seconds into the arrest period, the mouse was reattached to the ventilator and ventilated with 100% oxygen with a tidal volume of 150 µL and a respiratory rate of 160 breaths/ min. Eight minutes after injection of KCl, CPR was initiated by injection of 8 µg of warmed (37°C) epinephrine in 0.5 cc saline, via the jugular vein catheter, and chest compressions (approximately 300 per minute) were initiated. Until the mice were resuscitated, additional epinephrine was administered in increments of 0.5 µg every 30 seconds in conjunction with continued chest compressions until mice were resuscitated or until 32 µg epinephrine was administered. The total amount of epinephrine administered and the total duration of cardiac arrest/CPR time from KCl injection to the first minute of spontaneous mean blood pressure above 60 mmHg were recorded. Mice were maintained on 100% oxygen for 25 minutes after the return of spontaneous circulation and then extubated. This was followed by the removal of catheters and suturing of wounds. 0.75 cc of prewarmed lactated ringers was injected subcutaneously immediately following the conclusion of the procedure. Mice were placed in a clean cage on a thermal

barrier for an additional hour and given moistened food before being returned to the vivarium.

All the surgical procedures described above will be similar for all CA/CPR animals and SHAM. However, the SHAM animals do not receive KCl or EPI: a 0.5 mL injection of isotonic saline was administered instead. Therefore, the SHAM animals are not exposed to global ischemia, EPI, or chest compressions. Half of the SHAM animals in each experimental group had their brain maintained at 37°C, whereas the other half had their brains maintained at 27°C. It has been previously determined that decreasing the temperature of the brain to 27°C has no effect on the histological, physiological, or behavioral outcomes [13]. The SHAM groups were pooled if they did not differ in 1-way ANOVA and T-test comparisons. All surgeries were performed by a trained surgeon.

Histology

Mice were injected with an overdose of sodium pentobarbital and were transcardially perfused with 0.1 M PBS (4°C; pH 7.4), followed by 4% paraformaldehyde in 0.1 M PBS. When the brains were removed, they were immersed in 4% paraformaldehyde in 0.1 M PBS for a 4 hour post fix, cryoprotected in 30% sucrose in 0.2 M PB for 24 hours, frozen on dry ice and then stored at -70°C. Each slide contained approximately twelve brain sections cut at 12 µm on the cryostat, and the slides were saved for H&E and microglial staining (via anti-CD11b).

Microglial Activation Marker (Mac-1) Histochemistry (anti-CD11b)

Sections were rinsed with 0.2 M PB, immersed in hydrogen peroxide, and blocked with 4% rabbit serum in 0.5% Triton X-100 and 0.2 M PB. Slides were incubated for 24 hours at 4°C with anti-CD11b antibody (AbD Serotec, Raleigh, NC) which was diluted 1:200 in 0.2 M PB containing 0.3% Triton X-100 and 2% rabbit serum. Slides were then rinsed and incubated with biotinylated rabbit anti-rat secondary antibody (1:400; Vector Labs) for 2 h. Elite ABC in 0.2 M PB was then used to treat the slides for 30 min. Slides were again rinsed with 0.2 M PB and visualized with DAB containing nickel. Once visualization was complete, the slides were rinsed in distilled water for 2 min and dehydrated (through 50%, 75%, 80%, 90%, and absolute alcohol). Slides were then dipped ten times into xylene and coverslipped using Permount media. The activated microglia were counted in many microscopic fields through the hippocampus, caudate putamen, and the dentate gyrus.

Hematoxylin and Eosin Staining

Slides were dipped into tap water, and then immersed in Harris hematoxylin with 0.5% acetic acid for 2 minutes. Slides were then rinsed by dipping them into tap water 2 times. The slides were then treated with ammonia water (0.25%) until the brain sections turned blue followed by another 10 dips in water. Each slide was dipped in eosin 10 times and then dehydrated by dipping 10 times each 50%, 75%, 80%, 90%, and absolute alcohol. The slides were then cleared with 10 dips of xylene and cover slipped with Permount media.

Collapsed, pyknotic nuclei with pink eosinophyllic cytoplasm will be indicative of degenerating neurons. These structures will be counted throughout the CA1, CA2, CA3, and dentate gyrus regions of the hippocampus as well as the caudate/putamen.

Golgi Stain and Scholl Analysis

Freshly extracted mouse brains were treated according to the FD Rapid Golgistain Kit (FD NeuroTechnologies, Inc., Ellicott City, MD) protocol as directed by the manufacturer, immediately followed by counter staining with crestyl violet. For each region of interest, 5 neurons for each animal were outlined using Neurolucida (MBF Bioscience, Williston, VT), and a Scholl analysis was performed using the same software package to assay dendritic and axon length. Five different neurons per animal were selected, and dendritic spine density was counted using Neurolucida software.

Behavioral Testing

All behavioral testing occurred during the light phase of the circadian cycle. The individual conducting and scoring behavioral tests was uninformed of experimental assignments. The behavioral apparatuses were cleaned with 70% ethyl alcohol between each use. Only animals that completed the entire behavioral testing protocol were included in the statistical analysis

Locomotor activity

Locomotor activity was assessed in Flex Field photobeam activity systems (San Diego Instruments, San Diego, California, U.S.A.). Reduced anxiety-like behavior was indicated by more time spend in the center of the chamber. Additionally, under anxiogenic conditions, less exploring type behavior and rearing was observed. The apparatus was enclosed in individual sound attenuating chambers equipped with a 15-W fluorescent white light and ventilating fan that also provided masking noise. A clear Plexiglas insert (40 × 40 × 37.5 cm) was fitted inside a metal frame consisting of 16 equally spaced infrared photocell detectors. The photocells were located 2 cm from the floor along two adjacent walls of the chamber. Interruptions in the infrared light sources by the experimental animal were recorded by Photobeam Activity Software (PAS; San Diego Instruments, San Diego, CA). Beam breaks were converted to distance traveled (cm). Data were also analyzed to determine how much activity occurred in the periphery versus the center (a 90 cm² zone in the middle of the apparatus). Locomotor activity was assessed during 60-minute sessions at baseline and on post-surgical days before CA/CPR and day 4 of survival.

Elevated plus maze

The elevated plus maze was used as a measure of anxiety-like behavior. The apparatus consisted of two open arms and two closed arms arranged in a “+” orientation. The arms were 65 cm long and 5 cm wide. The walls enclosing

the closed arms were 15 cm high. The mouse was placed in the center of the apparatus facing an open arm, and the following measures were recorded: latency to enter arms, duration of time spent in closed and open arms, and frequency of arm entries. Reduced anxiety-like behaviors was indicated by increased open-arm entries, increased time spent in the open-arms, and decreased latency to enter the open arms. The 5-minute test was administered on the day prior to CA/CPR and day 4 of survival.

Restraint

Mice were placed in adequately ventilated clear polypropylene restrainers (50-mL conical tubes measuring 9.7 cm in length and internal diameter of 2.8 cm) for 2 h/d for. Animals were subjected to randomly timed (between 00:00 and 14:00 hr) restraint for two weeks prior to CA/CPR.

Statistical analysis

1-way ANOVA(s) were run for all stats and comparisons between groups was done using Tukey multiple comparison tests. Data were considered statistically significant if $p < 0.05$. When the data was not normally distributed or when variances were not equal across groups the non-parametric tests Kruskal-Wallis and Mann-Whitney U were used to analyze.

Results

Histology Group n's:

CA/CPR: n = 7 stress+CA/CPR: n = 8

SHAM: n = 6 stress+SHAM: n = 6

SHAM histology was not significantly different and therefore pooled

Post-Ischemic apoptosis in CA1 region of hippocampus

Hematoxylin and eosin stain (H & E) revealed significant increases in the number of degenerating cells in the CA1 region following CA/CPR relative to SHAM, and was further exacerbated with the addition of chronic stress (Fig.1). On the contrary, the CA2 region of the hippocampus did not show significance between the number of degenerating cells in the CA/CPR group relative to stress+CA/CPR group and overall there was less apoptosis in all models when compared to the CA1 region (Fig.2). The CA3 region showed near undetectable amounts of cell death and masked by error (results not shown). Overall, we observed significance in the total number of degenerating cells in the CA/CPR groups compared to the SHAM groups, but the addition of stress did not exacerbate the neuronal damage (Fig. 3). Similarly, there was no significance observed in the dentate gyrus and/or in the caudoputamen between the CA/CPR and stress+CA/CPR models (results not shown). These results suggest that chronic stress prior to cardiac arrest with cardiopulmonary resuscitation causes the most damage to the hippocampus in the CA1 region.

Post-Ischemic Mac1 expression

There was significant difference in the expression of Mac1 in the SHAM models when compared to the CA/CPR and stress+CA/CPR with the SHAM showing less expression. However, the activation of microglia was not exacerbated with the addition of stress to CA/CPR (Fig. 4) The CA2 region and the dentate gyrus showed a similar trend (results not shown). The CA3 region, however, displayed a larger trend of activated Mac1 cells in the CA/CPR models when compared to the stress+CA/CPR (Fig. 5). These results suggest the CA1, CA2 and dentate gyrus regions of the hippocampus do not express significant microglia activation with the addition of stress prior to CA/CPR.

Post-Ischemic mRNA Expression of Tumor Necrosis Factor- α in Hippocampus and Caudoputamen

There was a significant increase in the expression of post-CA/CPR TNF- α when compared to the SHAM counterparts in the hippocampus. No significance was observed in stress+post-CA/CPR compared to SHAM (Fig 6). There was a significant difference in the expression of TNF- α in the caudoputamen for the SHAM models when compared to the CA/CPR. These results were exacerbated with addition of stress (Fig 7). These results demonstrate the pro-inflammatory effects of glucocorticoids in the central nervous system and show that glucocorticoids sequester a stronger response in the caudoputamen.

Post-Ischemic mRNA expression of Interleukin-1 in the Hippocampus and Caudoputamen

Polymerase chain reaction for analysis of gene expression for IL-1 in the hippocampus showed a significant increase in the CA/CPR models compared to the SHAM counterparts. These results were not exacerbated with the addition of stress (Fig 8). In the caudoputamen, there was a significant increase in the expression of IL-1 in stress+CA/CPR when compared to both SHAM and CA/CPR. There was no significant increase in IL-1 between SHAM and CA/CPR (Fig 9).

Behavior Group n's

CA/CPR: n = 15 stress+CA/CPR: n = 19

SHAM: n = 11 stress+SHAM: n = 12

Hypo: n = 4 stress+Hypo: n = 3

Data from SHAM/hypothermic was pooled unless significance was found.

Total arm activity/percent open arm entry

Post-CA/CPR and post-stress+CA/CPR had significantly more total arm entries when compared to the post-SHAM, post-Hypo and post-stress+Hypo (Fig. 10).

This locomotor/hyperactivity is typical behavior after central nervous system damage. There were no significant differences observed in any pre- or post-groups in percent open arm entries. These data suggest that all groups exhibited anxiety-like responses (Fig. 11).

Total activity in open field

The post-CA/CPR and post-stress+CA/CPR had significantly more open field activity when compared to their respective cardiac arrest groups. Again, hyper-locomoter activity is indicative of brain damage (Fig. 12).

Activity in the center of the open field

The post-CA/CPR had significantly less percent activity when compared to the post-SHAM demonstrating that the CA/CPR mice have more anxiety-like responses (Fig. 13).

Golgi Stain group n's

No stress+CA/CPR: n = 8

No Stress+SHAM: n = 6

Chronic+CA/CPR: n = 11

SHAM: n = 5

Scholl analysis makes intersections of the branches around the cell body with concentric circles increasing in radius by 10 μ m diameter. Intersection frequency (number of branches) with these concentric circles looked near identical to dendritic length between intersection nodes. Because of this, I will only be referring to the apical arborization in this manuscript.

Scholl analysis of apical arborizations and length in the CA1 region

Scholl analysis revealed significant difference in the SHAM apical arborizations when compared to the CA/CPR and stress+CA/CPR with the SHAM having more arborizations in the CA1 region of the hippocampus (Fig. 14). Moreover, there is

a significant difference by distance in apical arborizations between the SHAM and stress+CA/CPR, but not between the SHAM and CA/CPR at two separate distance ranges: proximal and distal (Fig. 15). Additionally, the SHAM-CA/CPR produced a significant increase in the total arborizations in CA1 (apical + basal) relative to CA/CPR and stress+CA/CPR, but there was no effect of chronic stress on these measures (data not shown). These results suggest that neuronal branching and length shorten as a result of an ischemic injury however there is no significance with the addition of stress prior to the ischemic insult.

Spine density in the CA1/CA2 region

There was a significant decrease in both apical and basal spine density following CA/CPR relative to SHAM-CA/CPR, but was not further exacerbated with the addition of chronic stress (results not shown) in the CA1 region. The CA2 region showed a significant decrease in both apical and basal dendritic spine density relative to SHAM, but there was no effect of stress on these measures. However, there was a significant decrease in the total spine density (basal + apical) after CA/CPR and was exacerbated with the addition of stress (Fig. 16).

Discussion

In this study we examined the effects of chronic stress prior to cardiac arrest with cardiopulmonary resuscitation and its relation to post-ischemic behavior alterations, neuronal death, activation of microglia, gene expression of tumor necrosis factor α and Interleukin 1β , as well as alterations in both dendritic

branching and spine density. The results presented here confirm and extend the results of previous studies that demonstrated deleterious effects of global cerebral ischemia on the central nervous system. This is the first study that has directly assessed the exacerbation of these effects with the addition of stress.

Cardiac arrest with cardiopulmonary resuscitation in mice produces significant amounts of neuronal cell death in the CA1 region and was significantly exacerbated with the addition of stress. These data support the hypothesis that stress and glucocorticoids can exacerbate neuronal damage via necrosis and apoptosis. However, it was observed that the CA2, CA3, dentate gyrus, and caudoputamen regions did not increase the number of degenerating cells in response to stress. These results indicate that different regions of the hippocampus are more susceptible to brain damage due to an ischemic insult and stress can exacerbate this damage.

Previous studies have shown that necrotic cell death in the hippocampus can be influenced by a strong pro-inflammation response, such as the activation of microglia and release of various cytokines. We hypothesized that the addition of stress to an ischemic insult would exacerbate the immune response: increased release of TNF- α , IL-1, and activation of microglia. The Mac1 stain in the CA1 region of the hippocampus showed there was a significant difference in the expression of activated microglia between the CA/CPR and SHAM groups; however, there was no significant increase with the addition of stress. Additionally, there was virtually no cell death in the CA3 region but Mac 1 expression was observed. This suggests that activated microglia and

inflammation are not synonymous with cell death and moreover, not all regions react the same to an ischemic insult.

Polymerase chain reaction for analysis of TNF- α within the hippocampus displayed results that go against our hypothesis: significant difference between CA/CPR and SHAM groups, but there was no significance between stress+CA/CPR and the SHAM groups. Additionally, expression of IL-1 in the hippocampus showed significance between the SHAM and both CA/CPR groups, but was not exacerbated with the addition of stress.

However, PCR analysis indicated that the expression of TNF- α in the caudoputamen with the addition of stress significantly differed compared to the SHAM groups, but significant differences were not observed without the addition of stress to CA/CPR compared to the SHAM groups. Moreover, RNA expression for analysis of IL-1 was observed in the caudate, but there was significance between both CA/CPR groups. This finding raises the question of why TNF- α and IL-1 the caudate did not significantly differ when there was virtually no cell death or microglia activation. One possible speculation is that because the caudoputamen connects many regions of the brain it produces a signal that initiates the excitotoxic effects of glutamate observed in the hippocampus. Possibly, raised levels of cortisol exacerbate this signal but further research is required to answer that question.

The elevated plus maze and locomotor data presented demonstrate the increased locomotor and hyperactivity typically observed after damage to the central nervous system when compared to SHAM. However, the percent activity

in the center of the open field was the only behavioral test (besides percent central activity in the open-field) that demonstrated increases in anxiety-like responses following CA/CPR with the post-CA/CPR groups displaying significantly less percent activity than the post-SHAM. Because the percent open arm entries did not significantly increase with stress it is possible that the severe effects of an ischemic induced injury could mask the behavioral effects of the addition of stress.

It has been shown *in vitro* that both dendritic branches and spines are very sensitive to extracellular glutamate concentrations [4]. This amino acid causes changes in calcium homeostasis, and in the presence of toxic levels of glutamate, the dendritic spines and branches tend to retract [4]. As previously stated, global cerebral ischemia causes glutamate excitotoxicity and moreover, the glucose uptake inhibition of corticosterone can cause neuronal cell death and increased extracellular levels of glutamate as a result from an injury to the CNS [10]. Taken together, this would suggest that the addition of stress to CA/CPR would cause a significant decrease in dendritic arborization and density when compared to non-stress CA/CPR. This was observed in the present study. Scholl analysis of total apical arborizations, length, and spine density in the CA1 showed that SHAM has significantly more of each when compared to CA/CPR and stress+CA/CPR. Moreover, there were only differences between SHAM and stress+CA/CPR by distance in apical arborizations at two separate ranges from the cell body. There was also a wide range in which both CA/CPR groups significant differed from the SHAM group, but the two distances in which only the

stress+CA/CPR groups differed from the SHAM could indicate areas in which stress may have begun to exacerbate the dendritic retraction of the CA1 pyramidal cells. Because stress+CA/CPR was the only group that differed by distance relative to SHAM at these two ranges, it would suggest that elevated corticosterone concentration levels may be a key player in the retraction of dendritic branches and spines.

It is necessary to determine the role that stress-induced increases in corticosterone levels have in neuroinflammation and immune dysfunction following cardiac arrest with cardiopulmonary resuscitation. There has been recent evidence to suggest that innate and inflammatory immune responses increase the ischemic induced damage observed in the “watershed” regions of the brain. Glucocorticoids produce a stronger immune response in the CNS because they inhibit glucose uptake by the cell, thereby making cellular recovery from ischemic induced excitotoxicity more difficult by further reducing readily available energy sources [10]. This decreased metabolism coupled to ROS after reperfusion can have a major role in cellular injury and death. However, this alone is probably not sufficient to produce the exacerbated effects observed with the addition of stress to CA/CPR. One other possible key player involved in the cascade of events could be the activation of Toll-like receptors (TLRs); a family of signal transduction molecules that play a role in the immune response. Recent studies in murine models have shown that TLR4-mediated NF κ B pathway plays a role in the ischemia and reperfusion injury in the central nervous system [15].

The present study has shown that chronic stress can exacerbate the deleterious effects of global cerebral ischemia. Further research will be aimed at determining the role of stress-induced increases in glucocorticoids in ischemic outcome by giving injections to chronically stressed rodents with mifepristone: a GR antagonist. Moreover, if similar results are observed, using TLR4 knockout mice in addition to mifepristone could give further insight to the underlying mechanisms in exacerbated neuroinflammation and immune dysfunction following global cerebral ischemia.

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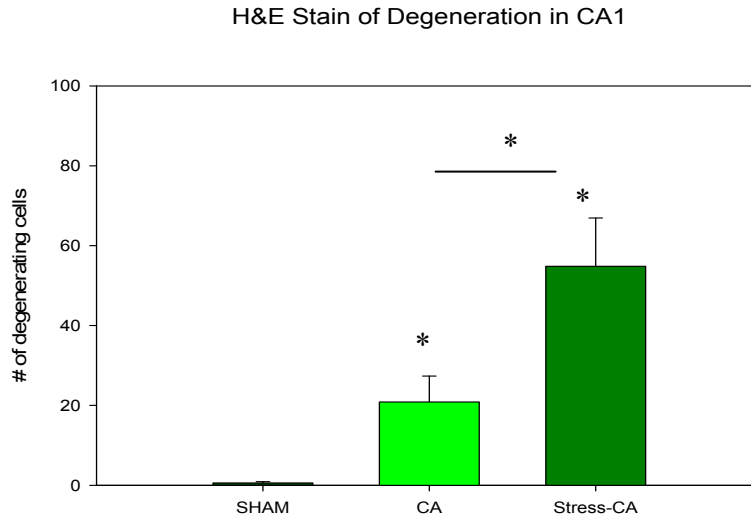


Figure 1: Post-Ischemic apoptosis in CA1 region shows a significant difference between number of degenerating cells between SHAM and CA/CPR and was further exacerbated with the addition of stress.

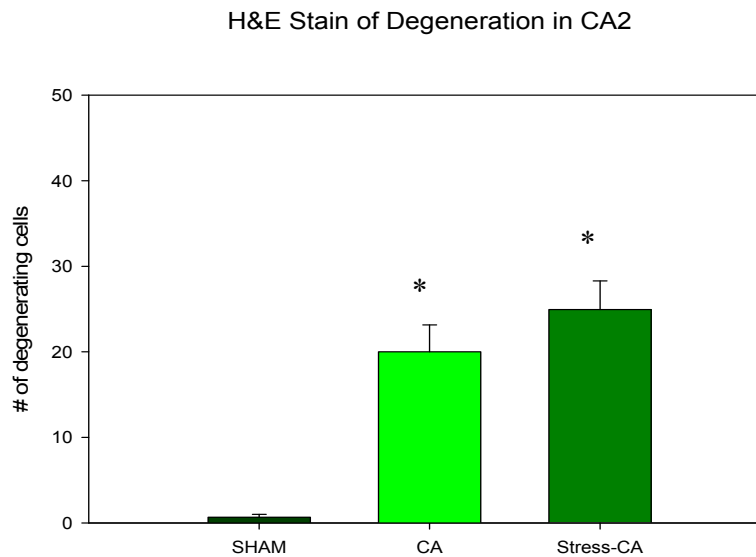


Figure 2: Post-Ischemic apoptosis in CA2 region shows a significant difference between number of degenerating cells between SHAM and CA/CPR, but was not further exacerbated with the addition of stress.

H&E Staining for Cell Degeneration in the Hippocampus

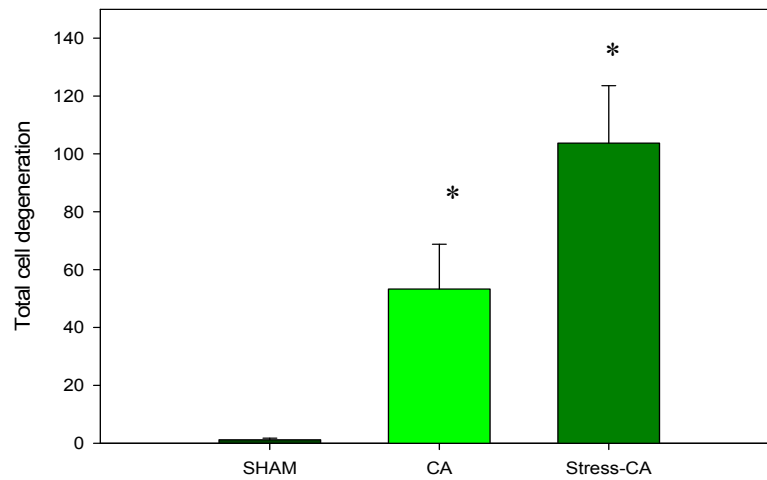


Figure 3: Total amount of post-ischemic apoptosis observed in the hippocampus. Significance observed between the CA/CPR and SHAM groups, but there was no effect of chronic stress on these measures.

Mac1 expression in CA1 of the Hippocampus

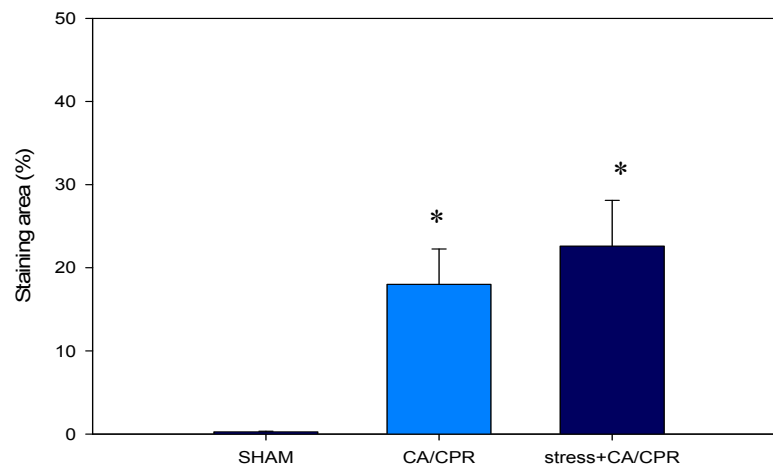


Figure 4: Microglia activation in the CA1 region of the hippocampus showed a significant increase between SHAM and CA/CPR. There was no effect of chronic stress on these measures.

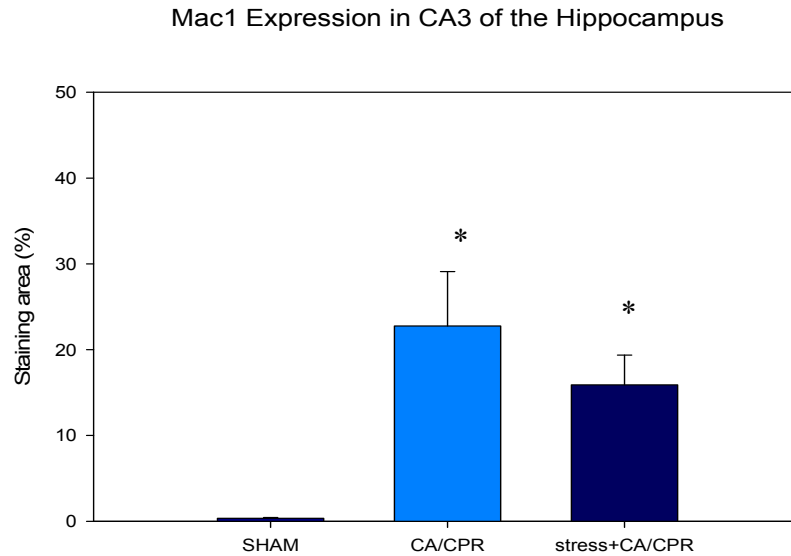


Figure 5: CA/CPR in the CA3 region displayed a larger trend of activated microglia than stress+CA/CPR. Both ischemic insults in the CA3 region displayed an overall less trend of Mac1 expression relative to CA1 region.

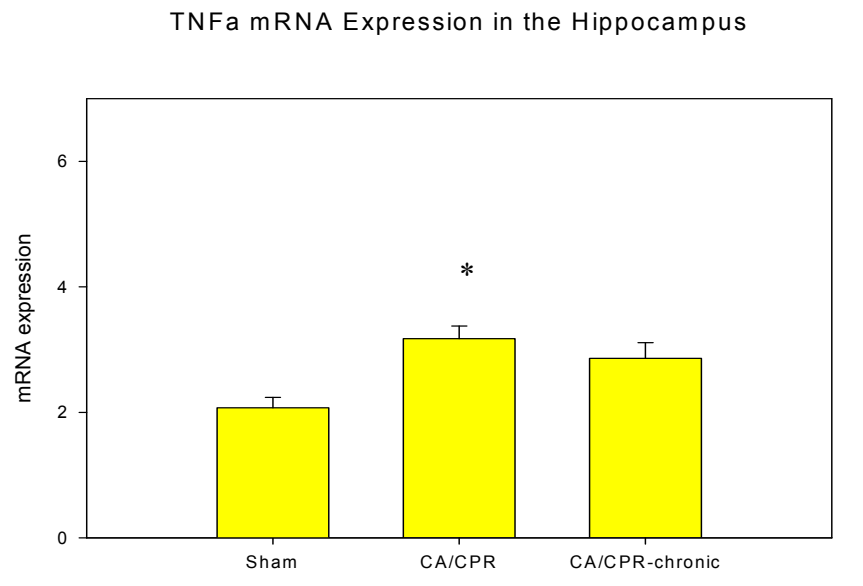


Figure 6: PCR analysis for expression of TNF – α in the hippocampus showed only a significant increase after CA/CPR.

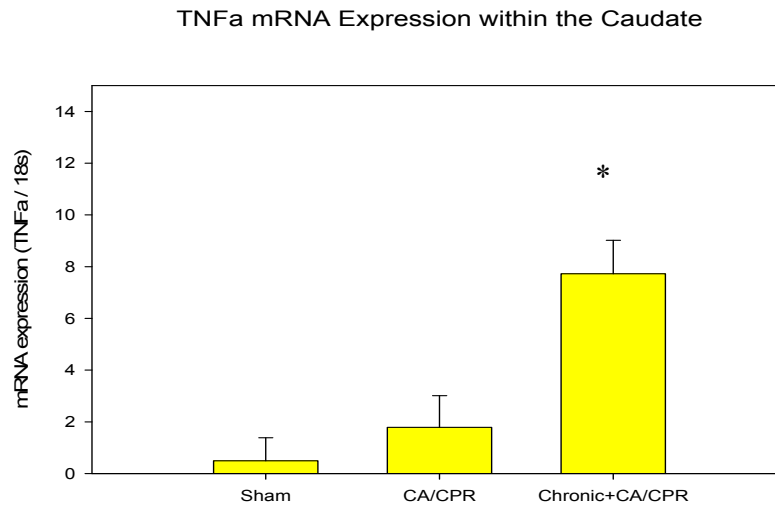


Figure 7: PCR analysis for expression of TNF – α in the Caudoputamen showed a significant increase only with the addition of stress.

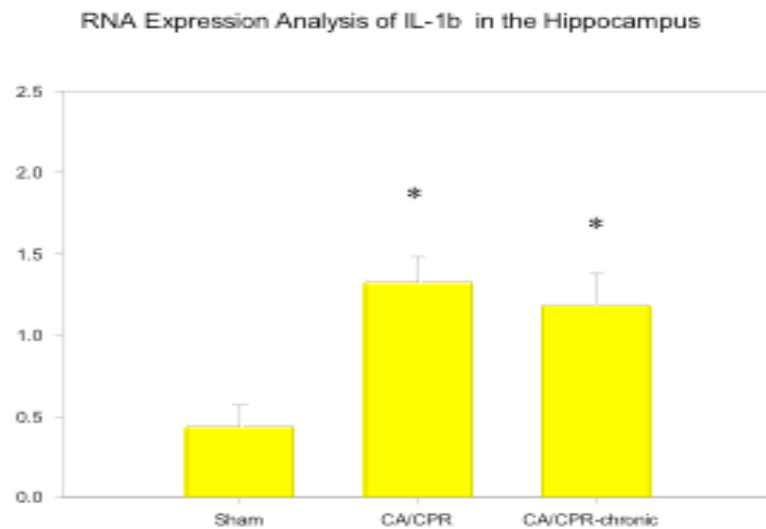


Figure 8: PCR analysis for expression of IL-1 in the hippocampus showed a significant difference between SHAM and CA/CPR, but was not exacerbated with the addition of stress.

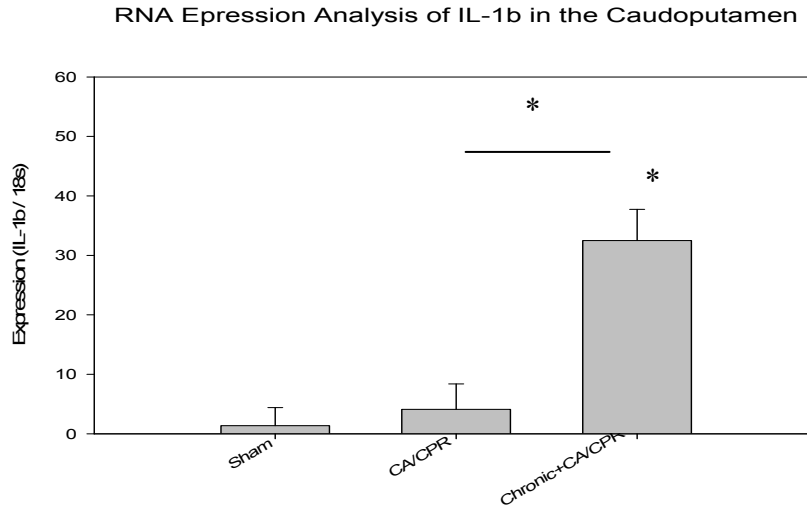


Figure 9: PCR analysis for expression of IL-1 showed a significant increase for stress+CA/CPR compared to SHAM and CA/CPR. There was no effect of CA/CPR on these measures.

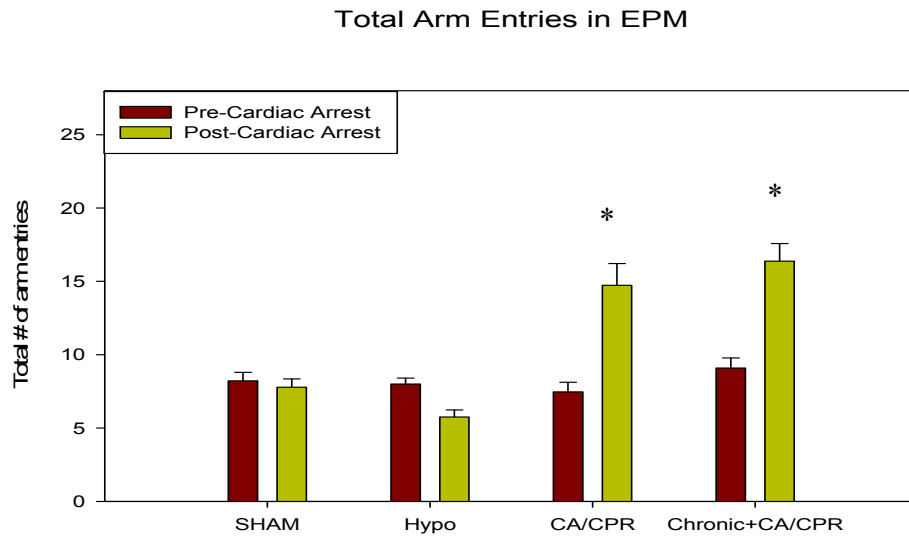


Figure 10: Post-CA/CPR and post-stress+CA/CPR had significantly more total arm entries compared to post-SHAM and post-hypo+CA/CPR.

Percent Open Arm Entries in EPM

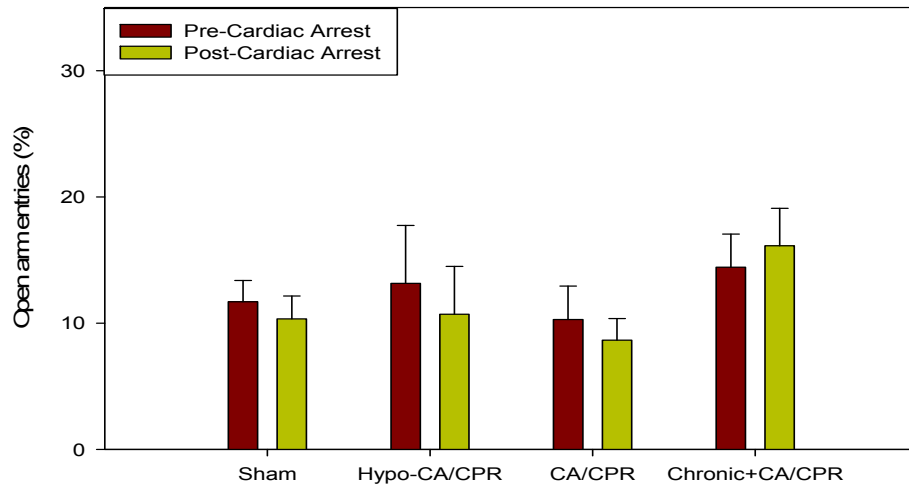


Figure 11: No significance was observed in any pre- or post- groups.

Total Activity in Open Field Test

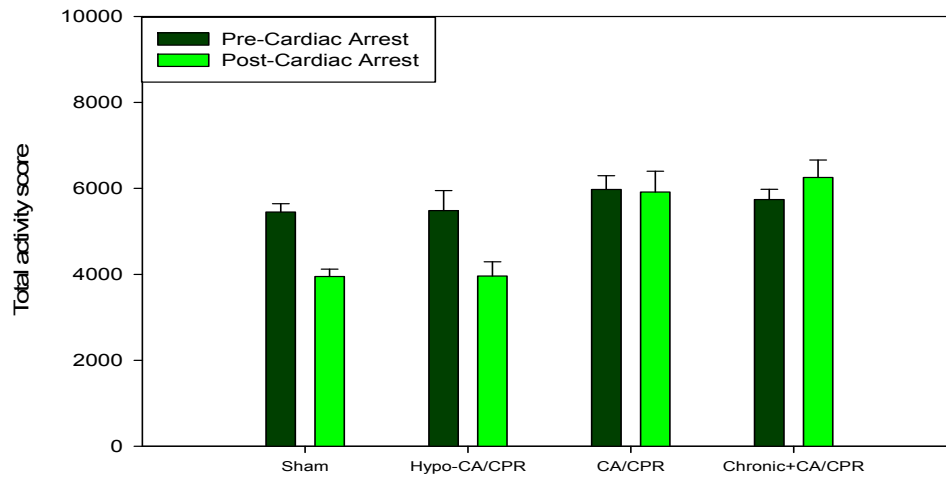


Figure 12: Post-CA/CPR and post-stress+CA/CPR had significantly more open field activity when compared to SHAM.

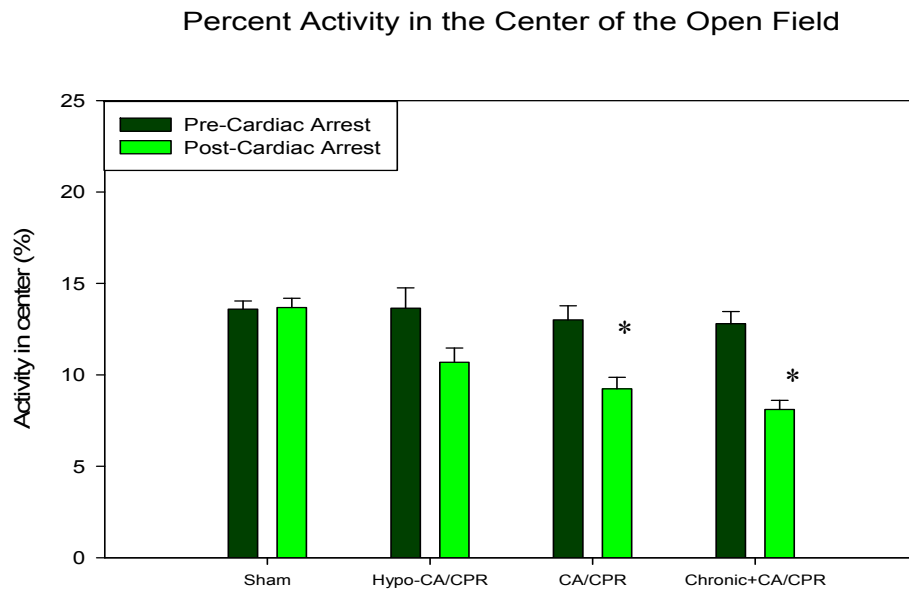


Figure 13: Post-CA/CPR and post-stress+CA/CPR had significantly less percent activity in the open field when compared to SHAM.

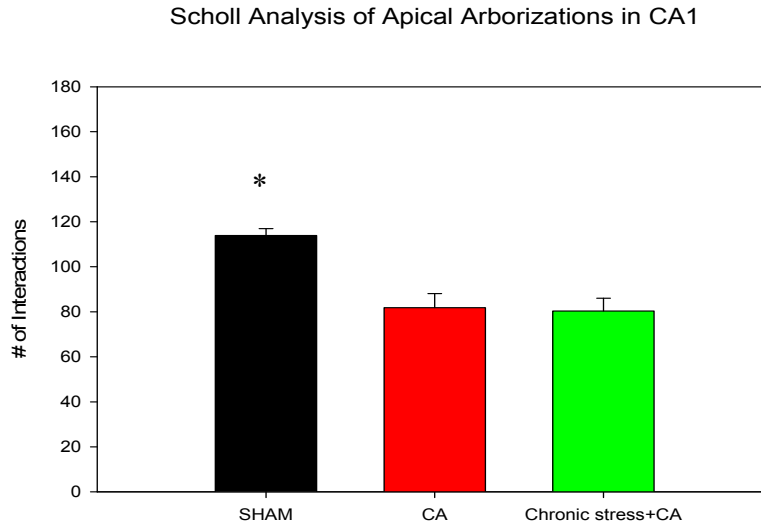


Figure 14: Scholl analysis showed a significant decrease in CA/CPR apical arborization relative to SHAM. There was no effect of chronic stress on these measures.

Apical Arborizations in the CA1 of the Hippocampus

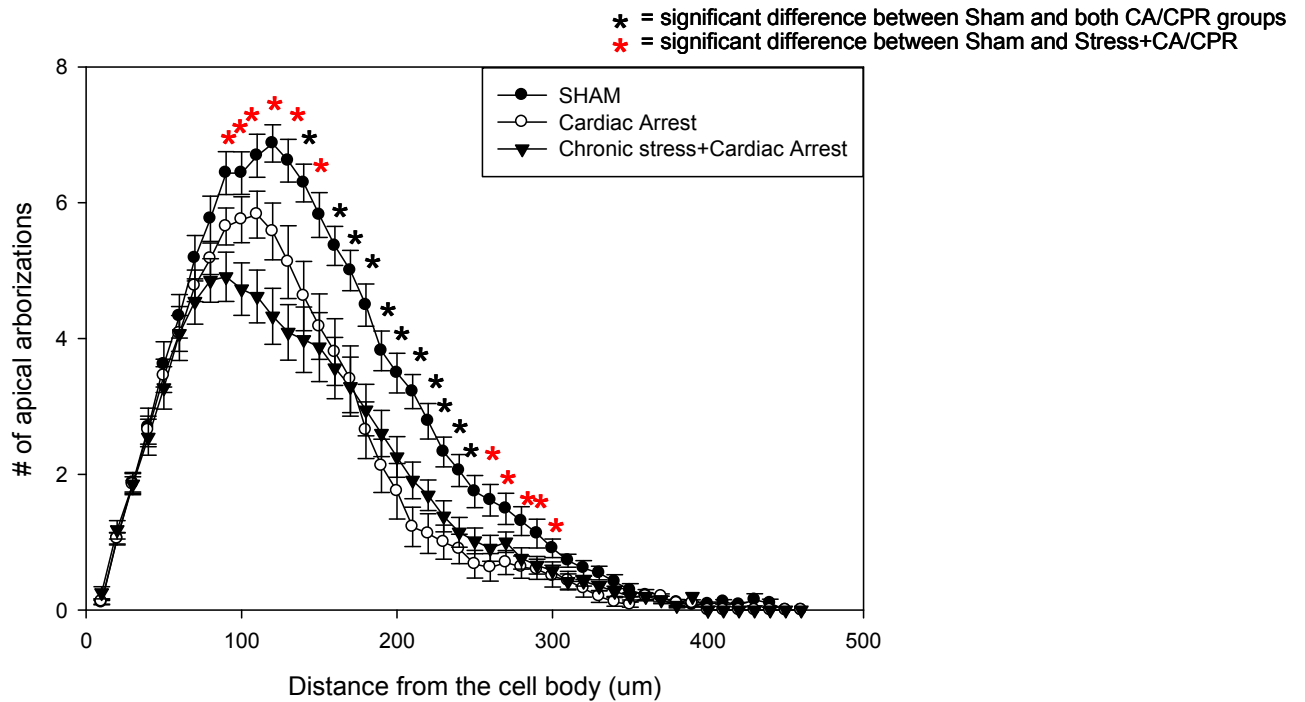


Figure 15: CA/CPR and stress+CA/CPR showed a significant decrease at varying distances in apical arborizations compared to SHAM.

Total Spine Density in the CA1 Region of the Hippocampus

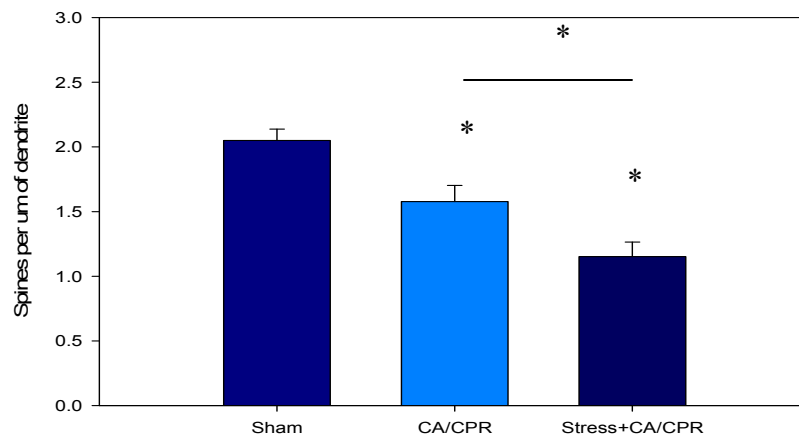


Figure 16: There was a significant decrease in total (apical +basal) spine density following CA/CPR relative to SHAM. This was exacerbated with the addition of stress.

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